Mycophenolic Derivatives from *Eupenicillium parvum*

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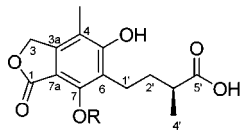
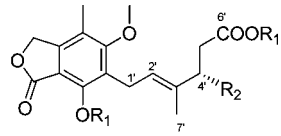
A new compound, euparvic acid (**1**, $C_{14}H_{16}O_6$), and the known compounds 5,7-dihydroxy-4-methylphthalide (**2**), 6-(3-carboxybutyl)-7-hydroxy-5-methoxy-4-methylphthalan-1-one (**3**), 6-(5-carboxy-3-methylpent-2-enyl)-7-hydroxy-5-methoxy-4-methylphthalan-1-one (**4**), and 6-(5-carboxy-4-hydroxy-3-methylpent-2-enyl)-7-hydroxy-5-methoxy-4-methylphthalan-1-one (**5**) were isolated from the EtOAc extract of *Eupenicillium parvum*. The structure of **1** was determined by interpretation of MS and homo- and heteronuclear 2D NMR spectroscopic data and confirmed by X-ray crystallography. The absolute configuration of **5** was determined via MPA ester derivatization.

Humate soil is organic matter that has a positive effect on the physical, chemical, and biological reactions of the soil, leading to an increase in soil fertility.[1] Humate is comprised of humic acid, fulvic acid, some minerals, bacteria, and fungi, which facilitate the development of deep root systems, enhance beneficial microbial activity, and improve overall plant health and resistance to diseases.[2]

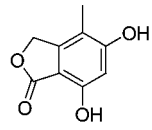
Continuing our earlier studies[3,4] on sourcing fungi for bioactive natural products, we have investigated the constituents of *Eupenicillium parvum*, isolated from a humate sample. Four known derivatives (**2**−**5**) of the antibiotic mycophenolic acid (MPA), previously isolated from the fermentation broths of several *Penicillium* species, and a new derivative (**1**) were found. Mycophenolic acid (**4**, $C_{17}H_{20}O_6$) is a potent inhibitor of human inosine 50-monophosphate dehydrogenase (IMPDH), a key enzyme in the *de novo* biosynthesis of guanine nucleotide. Its prodrug, mycophenolate mofetil (MMF), is used as an immunosuppressive agent to prevent kidney allograft rejection. MPA has also been shown to possess cytotoxicity against cancer cell lines.[5]



1 R = H
3 R = CH_3



4 R_1 = H; R_2 = H
5 R_1 = H; R_2 = OH
6 R_1 = CH_3; R_2 = OH



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We describe herein the isolation of five compounds (**1**−**5**) that were separated by column chromatography, Sephadex LH-20, and preparative TLC methods. While compound **1** is new, four compounds were previously identified as 6-(3-carboxybutyl)-7-hydroxy-5-methoxy-4-methylphthalan-1-one (**3**),[6] mycophenolic acid (**4**)[7] as a minor product, and 6-(5-carboxy-4-hydroxy-3-methylpent-2-enyl)-7-hydroxy-5-methoxy-4-methylphthalan-1-one (**5**).[8] The ^{1}H and ^{13}C NMR (Table 1) spectroscopic data of **3** are reported for the first time. The structure of 5,7-dihydroxy-4-methylphthalide (**2**) was confirmed by comparison of its spectroscopic data (MS, ^{1}H and ^{13}C NMR) with a literature reference.[9]

Table 1. ^{1}H−^{13}C NMR (δ, J in Hz in parentheses) Data[a] of Compounds **1** and **3**

	1[b]		**3**[b]	
	δ_H	δ_C[a]	δ_H	δ_C[a]
1		172.5 s		171.9 s
3	5.24 s	69.3 t	5.31 s	69.6 t
3a		144.8 s		145.3 s
4		110.3 s		116.7 s
5		161.1 s		163.6 s
6		114.8 s		122.3 s
7		153.3 s		153.5 s
7a		102.1 s		106.4 s
1′	2.82−2.85 m 2.74−2.76 m	20.6 t	2.75 dd (8.5)	21.2 t
2′	1.81−1.83 m 1.65−1.68 m	33.0 t	1.92−1.96 m 1.65−1.72 m	33.3 t
3′	2.41−2.44 m	38.9 d	2.47−2.54 m	39.0 d
4′	1.18 d (6.8)	17.0 q	1.22 d (7.3)	16.5 q
5′		179.3 s		176.9 s
CH_3-Ar	2.10 s	10.5 q	2.18 s	10.6 q
OCH_3			3.83 s	60.6 q

[a] Based on COSY, HSQC, HMBC, and ROESY experiments. [b] Spectrum recorded in acetone-d_6.

Compound **1** was characterized as 6-(3-carboxybutyl)-5,7-dihydroxy-4-methylphthalan-1-one, and the structure was determined on the basis of its MS and homo- (Table 1) and heteronuclear 2D NMR spectroscopic data and by X-ray crystallography.

Compound **1**, a white crystalline solid, mp 175−176 °C, showed a hydroxyl (3413 cm^{-1}) and a ketone group (1698 cm^{-1}) in the IR spectrum. The ESIQTOFMS yielded a parent mass at *m*/*z* 263.0919 corresponding to the pseudomolecular ion $[M - OH]^+$ (calcd $C_{14}H_{15}O_5$, *m*/*z* 263.0919). The ^{1}H NMR spectrum of **1** (Table 1) exhibited signals for a secondary methyl group at δ 1.18 (3H, d, J = 6.8 Hz), an aromatic methyl group at δ 2.10, and a singlet signal at δ 5.24 (2H) corresponding to an aliphatic methylene bearing oxygen. These data were closely comparable to those of the known compound 5,7-dihydroxy-4-methylphtalide (**2**)[9] and suggested that compound **1** contains this nucleus in its structure. The ^{13}C NMR (Table 1) and DEPT spectra of **1** disclosed 14 carbons, which were indicative of a saturated carboxylic acid at δ 179.3 (C-5′), two aliphatic methylenes at δ 20.6, and 33.0, an aliphatic methine at δ 38.9, and a secondary methyl group at δ 17.0, and suggested a chain in the position C-6 of the nucleus, in which the ^{1}H−^{1}H-COSY experiment allowed the sequential assignment. On the basis of the above data, **1** was identified as a derivative of mycophenolic acid,

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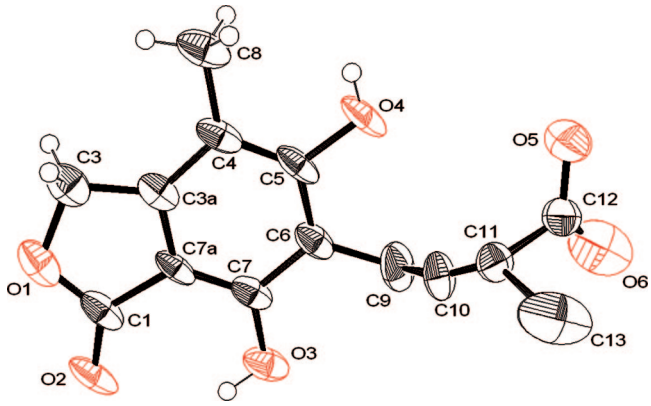

Figure 1. ORTEP-3 projection of compound **1**, with the displacement ellipsoids drawn at the 50% probability level. The atoms of the minor component of the disorder in the molecule and the hydrogens of both components have been omitted for clarity.

Table 2. ^{1}H NMR Shifts of the MPA Ester of Compound **6** before and after the Addition of the $Ba(ClO_4)_2$ Salt[a]

	δ_H MPA ester	δ_H MPA ester + $Ba(ClO_4)_2$	$\Delta\delta_{Ba}$
1′	3.293	3.192	+0.101
2′	5.530	5.528	+0.002
4′	5.241	5.166	+0.075
5′	2.673	2.679	–0.006
7′	1.612	1.528	+0.084

[a] 500 MHz, δ in ppm.

and its structure was established as 6-(3-carboxybutyl)-5,7-dihydroxy-4-methylphthalan-1-one (euparvic acid, **1**). The HMQC and HMBC spectra supported the structure assignments. Finally, the postulated structure was further validated by a single-crystal X-ray study (Figure 1).

With compound **3**, both the ^{1}H and ^{13}C NMR (Table 1) spectroscopic data are similar to those of **1**, but one difference in their ^{1}H NMR spectra was noted. An additional signal at δ 3.67 suggests the presence of a methoxy group at C-5. The ^{13}C (Table 1) and DEPT NMR spectra confirmed this observation. The structure was assigned and confirmed using HMBC and ROESY data. A literature search led to the identification of this compound as 6-(3-carboxybutyl)-7-hydroxy-5-methoxy-4-methylphthalan-1-one (**3**), isolated earlier from the fermentations of different fungi.[6]

Compound **5** exhibited NMR data (see Experimental Section) closely comparable to those of mycophenolic acid (**4**) (MPA),[10] except for the presence of a hydroxyl group attached to C-4′, corresponding to the structure 6-(5-carboxy-4-hydroxy-3-methylpent-2-enyl)-7-hydroxy-5-methoxy-4-methylphthalan-1-one. However, the relative configuration at that center (C-4′) was not reported. The absolute stereochemistry was assigned for **5**, using Riguera's method for the determination of the absolute stereochemistry of secondary alcohols.[11] Thus, compound **5** was converted to a methyl ester (**6**) with $TMSCHN_2$, which also methylated the phenol group. Then, the methyl ester was treated with DMAP, DCC, and (*S*)-MPA reagent in dry CH_2Cl_2. The ^{1}H NMR and COSY spectra of the resulting MPA esters were recorded in acetonitrile-d_3. Next, solid anhydrous $Ba(ClO_4)_2$ was added to the NMR tube until saturation and new ^{1}H NMR and COSY spectra were recorded. The chemical shifts before and after the addition of the barium salt to the MPA ester of **6** are summarized in Table 2. As a result of the addition of the barium salt, the more stable conformation was favored and the NMR signals of the 5′ protons were shifted to a lower field (negative $\Delta\delta^{Ba}$ values). In contrast, protons 1′, 2′, and 7′, which belong to the L_1 group, were shifted to the higher field ($\Delta\delta^{Ba}$ L_1 > 0 and $\Delta\delta^{Ba}$ L_2 < 0). On the basis of the experimental results obtained, the *S* absolute configuration was assigned to C-4′ (Figure 2), so the structure of compound **5** was

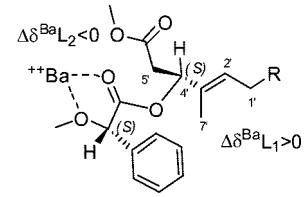

Figure 2. Ba^{2+} complex of the (*S*)-MPA ester of **6**.

established as 6-[(4*S*)-5-carboxy-4-hydroxy-3-methylpent-2-enyl]-7-hydroxy-5-methoxy-4-methylphthalan-1-one.

Compounds **1**−**6** did not show activity in standard disk assays against *Escherichia coli* (ATCC 35218), *Pseudomonas aeruginosa* (ATCC 27853), methicillin-resistant *Staphylococcus aureus* (ATCC 43300), *Mycobacterium intracellulare* (ATCC 27853), or *Aspergillus fumigatus* (ATCC 27853). Only compound **4** showed moderate activity [IC_{50} μM: 8.0 and 15.0] against *Candida albicans* (ATCC 90028) and *Cryptococcus neoformans* (ATCC 90113), respectively.

Experimental Section

General Experimental Procedures. The melting point was determined on a Opti-Melt MPA 100 instrument (Stanford Research Systems) and is uncorrected. Optical rotations were measured using a Rudolph Research Analytical Autopol V polarimeter. IR spectra were recorded using a Perkin-Elmer model spectrum-100 spectrophotometer. ^{1}H and ^{13}C NMR spectra were obtained on Bruker model AMX 500 and 400 NMR spectrometers with standard pulse sequences, operating at 500 and 400 MHz in ^{1}H and 125 and 100 MHz in ^{13}C. Acetone-d_6, CD_3OD, and CD_3CN were used as solvents, and TMS was used as internal standard. High-resolution mass spectra (HRMS) were recorded on a Micromass Q-Tof Micro mass spectrometer with a lock spray source. Column chromatography was carried out on silica gel (70−230 mesh, Merck). Fractions obtained from column chromatography were monitored by TLC (silica gel 60 F_{254}), and preparative TLC was carried out on silica gel 60 $PF_{254+366}$ plates (20 × 20 cm, 1 mm thick).

Fungal Material. The particular humate used was collected in Cuba, NM, in March 2008 and was plated out on potato-dextrose agar (PDA) that was maintained at 24 °C, until discrete fungal colonies appeared. Samples were taken from colonies and kept on PDA slants in test tubes at 24 °C, then placed in a 4 °C refrigerator until used. The fungus was keyed to *Eupenicillium parvum* by comparison of its morphological and DNA profile data with the library in the National Centre for Advanced Technologies, Lincoln University, New Zealand. A voucher specimen (UM-042106) has been deposited in the culture collection of the Medicinal Chemistry Department, University of Mississippi. The fungus was inoculated in 50 mL of potato-dextrose broth and kept for two weeks in stationary phase at 25 °C; then the mycelium and spore-laden broth (1 mL) were seeded onto a medium in each flask consisting of 100 g of shredded wheat, 200 mL of low-pH Oxoid mycological broth, 2% yeast extract, and 20% sucrose in 2.8 L Fernbach flasks (22 flasks) followed by incubation for 22 days at 24 °C.

Extraction and Isolation. Following incubation, 300 mL of acetone was added to each flask, and the mycelia and the substrate were homogenized (Super Dispex, Tekmark Co., SD-45). The suspension was filtered and the filtrate concentrated under a vacuum at <50 °C. The residue was mixed with H_2O (200 mL), then extracted with EtOAc (500 mL × 3). The combined EtOAc extracts were dried over anhydrous Na_2SO_4 and concentrated under a vacuum. The EtOAc extract (7.0 g) was chromatographed on silica gel 60, 70−230 mesh (400 g), with fractions stepwise eluted with CH_2Cl_2−Me_2CO mixtures (8:2; 7:3; 1:1), Me_2CO, and Me_2CO−MeOH (9:1) (each 200 mL), yielding four fractions. Fraction 1 (150 mg) was rechromatographed over silica gel, eluted with $CHCl_3$−EtOAc (9:1), to yield 5,7-dihydroxy-4-methylphtalide (**2**, 50 mg) and mycophenolic acid (**3**, 12 mg). Fraction 2 (200 mg) and fraction 3 (350 mg) were chromatographed over a silica gel 60 column and eluted with a $CHCl_3$−MeOH gradient to yield five subfractions. Subfractions 3−5 were chromatographed by preparative TLC with $CHCl_3$−MeOH (19:1) three times, affording 6-(3-carboxybutyl)-7-hydroxy-5-methoxy-4-methylphthalan-1-one (**4**, 10 mg) and 6-(3-carboxybutyl)-5,7-dihydroxy-4-methylphthalan-1-one (**1**, 8 mg). From the last fraction, a solid was recovered and purified on a silica gel column eluted with $CHCl_3$−MeOH (50:1), followed by preparative

TLC with CH_2Cl_2−MeOH (7:3), affording 6-(5-carboxy-4-hydroxy-3-methylpent-2-enyl)-7-hydroxy-5-methoxy-4-methylphthalan-1-one (**5**, 30 mg).

Euparvic acid (1): colorless solid (MeOH−EtOAc); mp 175−176 °C; $[\alpha]^{20}_D$ −4.0 (*c* 0.01, MeOH); IR (film) ν_{max} 3413, 2930, 1698, 1629, 1446, 1333, 1179, 1025, 932, 782 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; ESIQTOFMS *m/z* 280 (M^+, absent), 263 (100), 235 (100); HRESITOFMS *m/z* 263.0924 $[M - OH]^+$ (calcd for $C_{14}H_{15}O_5$, 263.0924), 235.0974 $[M - CO_2H]^+$ (calcd for $C_{14}H_{15}O_5$, 235.0970).

X-ray Crystallographic Study of Euparvic Acid (1). A single-crystal X-ray diffraction study was conducted on 6-(3-carboxybutyl)-7-hydroxy-5-methoxy-4-methylphthalan-1-one (**1**). Colorless needles were obtained with the slow evaporation of a solution in methanol. A single crystal, approximate dimensions 0.15 × 0.09 × 0.05 mm, was used for data collection on a Bruker Smart Apex II system,[12] using Cu Kα radiation with a graphite monochromator, fine-focus sealed tube. The crystal was kept at 100 K under a stream of cooled nitrogen gas from a KRYO-FLEX low-temperature device. Compound **1**, $C_{14}H_{16}O_6$, MW = 280.27, crystallizes in the monoclinic space group $P2_1/n$, with one molecule in the asymmetric unit and four molecules per unit cell ($Z = 4$). Cell dimensions are a = 12.5306(8) Å, b = 6.8656(3) Å, c = 15.7783(9) Å, β = 105.403(4)°, V = 1308.65(13) $Å^3$. Data collection, indexing, and initial cell refinements were all carried out using APEX II software. All non-hydrogen atoms were visible in the difference electron density maps. Anisotropic displacement parameters were included in the refinement for all non-hydrogen atoms. Frame integration and final cell refinements were done using SAINT software;[13] 8451 reflexions were read, with 397 rejected. The final cell parameters were determined from least-squares refinement on 1822 reflections, with R_{int} = 0.050 and $wR(F^2)$ = 0.084, and GooF = 1.024. The maximum resolution of the data collection was 0.85 Å. Most of the aliphatic side chain, C9, C10, C11, C12, C13, O5, and O6, was disordered. An attempt made to model disorder in this region during refinement allowed for the modeling of four disordered pairs, C10−C10a, C11−C11a, C12−C12a, and O5−O5a. The average population ratio of the disordered pairs was 0.413 to 0.587. In C9, C13, and O6, the disordered pairs were too close to be separated after several refinement cycles and were left as large ellipsoids in the final projection.

Structure solution, refinement, graphics, and generation of publication materials were performed using SHELXTL, V6.12 software.[14] Hydrogen atoms were placed in their expected chemical positions using the HFIX command and were included in the final cycles of least-squares refinement, with isotropic U_{ij}'s related to the atoms ridden upon. The crystallographic data have been deposited in the Cambridge Crystallographic Data Centre (deposition number 684326), and the data can be obtained free of charge from http://www.ccdc.cam.ac.uk/deposit.

6-(3-Carboxybutyl)-7-hydroxy-5-methoxy-4-methylphthalan-1-one (3): amorphous solid; $[\alpha]^{20}_D$ −2.8 (*c* 0.01, MeOH); IR (film) ν_{max} 3232, 2930, 1696, 1614, 1445, 1409, 1372, 1325, 1211, 1160, 1134, 1106, 1079, 1036, 962, 816, 795 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; ESIQTOFMS *m/z* 317 $(M + Na)^+$, 307 (20), 295 (25); HRESITOFMS *m/z* 317.1006 $[M + Na]^+$ (calcd for $C_{15}H_{18}O_6Na$, 317.1001).

6-(5-Carboxy-4-hydroxy-3-methylpent-2-enyl)-7-hydroxy-5-methoxy-4-methylphthalan-1-one (5): amorphous solid; IR (film) ν_{max} 3365, 2938, 2865, 1695, 1557, 1405, 1323, 1263, 1194, 1136, 1056, 1032, 970, 728 cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz) δ 1.63 (3H, s, CH_3-7′), 1.97 (3H, s, CH_3-Ar), 2.10 (2H, s br, H_2-5′), 3.20 (2H, d, J = 5 Hz, H_2-1′), 3.61 (3H, s, OCH_3), 4.07 (1H, s br, H-4′), 5.13 (2H, s, H_2-3), 5.37 (1H, dd, J = 5.4 Hz, H-2′); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 11.5 (CH_3-Ar), 12.5 (C-7′), 22.7 (C-1′), 43.0 (C-5′), 60.6 (OCH_3), 69.3 (C-3), 73.6 (C-4), 106.8 (C-7a), 110.0 (C-4), 123.2 (C-2′), 124.2 (C-6), 136.2 (C-3′), 145.3 (C-3a), 153.0 (C-7), 163.6 (C-5), 171.0 (C-1), 178.1 (C-6′); ESIQTOFMS *m/z* 359 $(M + Na)^+$, 319 (60), 301 (40), 293 (20); HRESITOFMS *m/z* 359.1112 $[M + Na]^+$ (calcd for $C_{17}H_{20}O_7Na$, 359.1107).

Methyl Ester of 5 (6). To solution of **5** (3.0 mg) in CH_2Cl_2 (2.0 mL) was added excess $TMSCHN_2$ (1 mL, 2.0 M), and the mixture stirred at room temperature for 48 h. The reaction mixture was concentrated under vacuum and purified by preparative TLC (silica gel: benzene−EtOAc, 7:3) to furnish 3.0 mg of **6**: ^1H NMR ($CDCl_3$, 500 MHz) δ 1.81 (3H, s, CH_3-7′), 2.17 (3H, s, CH_3-Ar), 2.53 (2H, m, H_2-5′), 3.43 (2H, d, J = 3.4 Hz, H_2-1′), 3.67 (3H, s, OCH_3), 3.77 (3H, s, OCH_3), 4.04 (3H, s, OCH_3), 4.42 (1H, d, J = 4.4 Hz, H-4′), 5.13 (2H, s, H_2-3), 5.49 (1H, dd, J = 5.5 Hz, H-2′); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 11.5 (CH_3-Ar), 12.15 (C-7′), 23.08 (C-1′), 39.98 (C-5′), 51.81 (OCH_3), 61.04 (OCH_3), 62.70 (OCH_3), 68.37 (C-3), 73.16 (C-4′), 112.52 (C-7a), 119.98 (C-4), 124.94 (C-2′), 128.32 (C-6), 135.74 (C-3′), 146.91 (C-3a), 156.78 (C-7), 162.79 (C-5), 168.92 (C-1), 173.00 (C-6′); HRESITOFMS *m/z* 387.1422 $[M + Na]^+$ (calcd for $C_{19}H_{24}O_7Na$, 387.1420).

Preparation of the (*S*)-MPA Ester of Compound 6. A 2.0 mg (0.0054 mmol) sample of the methyl ester **6** was treated with (*S*)-methoxyphenylacetic acid (2.0 mg, 0.012 mmol), DCC (8.0 mg, 0.038 mmol), and DMAP (0.8 mg, 0.006 mmol) in DCM (10 mL) for 48 h at room temperature. The reaction mixture was worked up, and the resulting MPA ester of **6** (0.7 mg) was dissolved in CD_3CN for ^1H NMR measurements. HRESITOFMS: *m/z* 535.1948 $[M + Na]^+$ (calcd for $C_{28}H_{32}O_9Na$, 535.1944).

Bioassays. Compounds **1** and **3**−**6** were tested against the microorganisms *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, methicillin-resistant *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Mycobacterium intracellulare*. Amphotericin A was used as positive control for the first three microorganisms, and ciprofloxacin was used for the others. Procedures for most of the antimicrobial susceptibility assays were performed using a modified version of the NCCLS methods,[15] while the susceptibility testing against the *Mycobacterium* was done using the modified Alamar Blue procedure of Franzblau et al.[16] Samples were dissolved in DMSO and were serially diluted using 0.9% saline. These were transferred in duplicate to 96-well microplates. Plates were read at either 630 nm or 544ex/590em (*M. intracellulare*) prior to and after incubation. Percent growth was plotted versus test concentration to afford the IC_{50}.

While the biological activities in these systems are limited in scope, attempts to isolate suitable quantities of euparvic acid will be performed to test its effects in a broader range of bioassays.

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